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Short Communication

Systematic Reverse Genetic Screening of T-DNA Tagged Genes in Rice for Functional Genomic Analyses: MADS-box Genes as a Test Case

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We have generated 47 DNA pools and 235 subpools from 21,049 T-DNA insertion lines of rice. DNA pools of 500–1,000 lines were adequate for screening a T-DNA insertion within a 2-kb region. To examine the efficacy of the DNA pools, we selected MADS-box genes, which play an important role in controlling various aspects of plant development. A total of 34 MIKC-type MADS-box genes have now been identified from rice sequence databases. Our PCR screening for T-DNA insertions within 12 MADS-box genes resulted in the identification of five insertions in four different genes. These DNA pools will be valuable when isolating T-DNA insertional mutants in various rice genes. The DNA pool screening service and the mutant seeds are available upon request to genean@postech.ac.kr.

Keywords: Insertional mutagenesis — MADS-box genes — Pool screening — Rice — T-DNA.

Because sequencing of the rice genome is nearly completed (Goff et al. 2002, Sasaki et al. 2002, Yu et al. 2002b), the determination of gene function is now a most challenging goal. To this end, several reverse genetic approaches have been developed, including homologous recombination (reviewed in Hanin and Paszkowski 2003), anti-sense or RNAi suppression (Chuang and Meyerowitz 2000), and insertional mutagenesis (Feldmann 1991, Jeon et al. 2000a). Among these methods, random insertional mutagenesis by transposons or T-DNA have been most widely used for large-scale analyses. This technique is not only efficient, but can also be employed for promoter trapping and activation tagging. The establishment of a large number of insertional mutants in *Arabidopsis* has accelerated the reverse genetics approach (Feldmann 1991, Azpiroz-Leehan and Feldmann 1997, Krysan et al. 1999, Parinov et al. 1999, Parinov and Sundaresan 2000, Sessions et al. 2002, Szabados et al. 2002).

For functional analyses of insertional-mutant lines, it is essential that one be able to easily identify disrupted genes. One method is to establish the flanking sequence database of the insertion sites. In one study, for example, flanking sequences were analyzed from 932 independent *Dissociation* transposant lines, and a 1,200 insertion-site database was developed from the nonautonomous defective *Suppressor-mutator* lines in *Arabidopsis* (Parinov et al. 1999). Moreover, 85,108 insertion sequences from 52,964 T-DNA lines were isolated using the high-throughput modified thermal asymmetric interlaced (TAIL)-PCR protocol (Sessions et al. 2002) (www.tmri.org). In rice, the *Tos17* insertion sequence database also has been constructed (Hirochika 2001, Yamazaki et al. 2001). From these insertion sequence databases, one can readily identify knockout mutants in a gene of interest. However, establishing such a database requires much time and effort.

As an alternative, insertional mutants can be identified through a pooling strategy. In this method, PCR analyses of DNA pools from several hundred to a thousand mutant lines enable researchers to locate those insertional mutants without knowing the flanking sequences of each position. This strategy has been successfully applied with *Arabidopsis*, petunia, and maize (Koes et al. 1995, Krysan et al. 1999, Mena et al. 1996, Parinov and Sundaresan 2000). For example, 17 insertions in 63 genes involved in signal transduction and ion transport, 47 insertions in 36 members of the R2R3 MYB gene family, and 22 mutations in 70 P450 genes were isolated from *Arabidopsis* (Krysan et al. 1996, Meissner et al. 1999, Winkler et al. 1998). PCR-based reverse genetic screenings of the *Ac* and *Tos17* insertion lines in rice have been reported as well (Enoki et al. 1999, Hirochika 2001). Of 14 randomly selected genes, two knockouts were identified from the *Ac* pools made from 6,000 individuals (Enoki et al. 1999). One knockout mutant in *OSH15* was isolated from 47 DNA pools consisting of an average of 550 individuals (Sato et al. 1999). Three independent phytochrome-A mutants were also found from *Tos17* pools (Takano et al. 2001).

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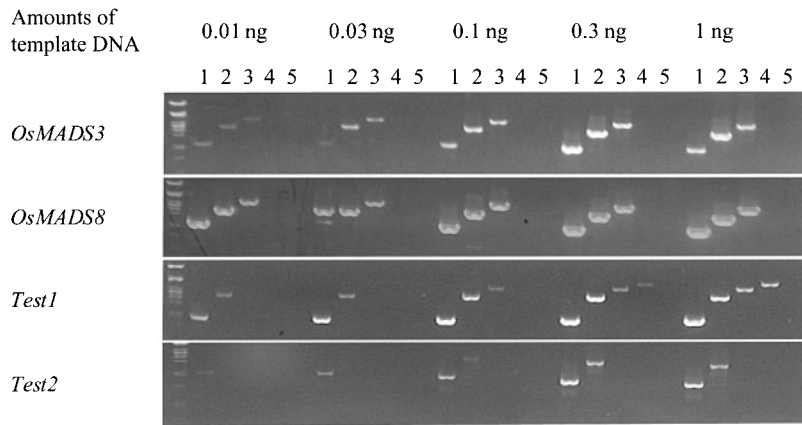
Fig. 1 Template detection limit and targeting range of PCR. Various amounts of genomic DNA templates (0.01, 0.03, 0.10, 0.30, or 1.00 ng) of four tagged genes (*OsMADS3*, *OsMADS8*, *Test1*, and *Test2*) were used for PCR analyses in which a T-DNA specific primer and five gene-specific primers were located about 1, 2, 3, 4, or 5 kb from the T-DNA insertion sites.

Because *Tos17* and *Ac* insertions appear to have hot spots, alternative methods, e.g. T-DNA insertional mutagenesis, will be valuable for complementing the transposon taggings (Yamazaki et al. 2001). We have previously reported the establishment of a large number of T-DNA insertional mutants in rice (Jeon et al. 2000a, Jeong et al. 2002). The transgenic plants contain an average of 1.4 loci of T-DNA inserts (Jeon et al. 2000a). Here, we report construction of DNA pools from T-DNA mutant lines. We also elucidate the usability of this pool by screening knockout mutants in the MADS-box genes, which are involved in various aspects of plant development (reviewed in Theissen et al. 2000).

Before we could make these DNA pools, we had to determine the optimum pool size. Therefore, a stringent PCR annealing temperature (65°C) and long primers (25–30 bp) were employed because these allowed more individual lines to be pooled (Krysan et al. 1996, Krysan et al. 1999). PCR consisted of 96°C for 5 min, followed by 36 cycles of 95°C for 15 s, 65°C for 30 s, 72°C for 2 min; and finally 72°C for 7 min. Several analyses were done to set the proper screening conditions. As test cases, we selected four genes, into which T-DNA was inserted. Two of them encode for OsMADS3 and OsMADS8 (Kang et al. 1995, Kang et al. 1997). The other two, with unknown functions, were designated as *Test1* (GenBank accession number D22254) and *Test2* (GenBank accession number BAA94238). Within the 5-kb regions from the T-DNA insertion points, the total GC contents of the first three genes (*OsMADS3*, *OsMADS8*, and *Test1*) were 34%, 37%, and 38%, respectively, which are lower than the GC content of the rice genome (Yu et al. 2002b). In contrast, the GC content of *Test2* was 46%, which is higher than average.

To examine the detection range of our PCR analysis, we designed five gene-specific primers, located 1, 2, 3, 4, and 5 kb from the T-DNA insertion sites. We also determined the minimum amount of template required for screening. PCR products of up to 3 kb could be detected for the *OsMADS3* and *OsMADS8* genes at all template levels evaluated (Fig. 1). However, amplification efficiency was lower for *Test1*. Product was

not detectable when the template amount was reduced below 0.1 ng and the primer site was located at >2.0 kb. Unlike the first three genes, the PCR products for the *Test2* gene were hardly amplified, and our results were inconsistent under standard reaction conditions (data not shown). This was probably due to a higher level of GC in the gene. Because betaine has been successfully used in the amplification of GC-rich regions (Hengen 1997, Henke et al. 1997), we examined its effect here in rice. PCR products were amplified reproducibly for *Test2* when 0.8 M betaine was added to the reaction mixture (Fig. 1). However, both efficiency and the targeting range were low compared with the first three genes, all of which contained lower levels of GC. We also observed that an annealing temperature of 58–60°C was related to the most efficient amplification when 0.8 M betaine was added (data not shown). These results indicate that special care is needed when screening knockout mutants in rice genes because a significant proportion consists of high GC levels (Yu et al. 2002b).

For screening a knockout from the DNA pools of *Arabidopsis*, 50–100 ng of template DNA has been used (Galbiati et al. 2000, Meissner et al. 1999, Winkler et al. 1998). Because 0.1 ng of template DNA is required for rice, we estimated that a DNA pool of 500 to 1,000 individuals would be adequate. To test whether this was correct, we made DNA pools composed of 100, 300, 500, 1,000, and 1,500 independent T-DNA tagged lines. In each pool, we included the four lines that carry T-DNA insertions in the *OsMADS* genes or *test* genes. PCR amplification of target fragments in the DNA pools showed that insertions within *OsMADS8* could be detected from all pool sizes (Fig. 2). However, signals were not as strong when the amplified fragment was longer than 3 kb. For the three other genes, hybridization signals were weaker especially when the insertion sites were located beyond 2 kb. Furthermore, hybridization intensity decreased as the pool size enlarged (Fig. 2).

In *Arabidopsis*, Krysan et al. (1999) set the maximal size at ~2,350 lines per pool, whereas Winkler et al. (1998) reported that pool sizes of 600 to 1,000 lines were acceptable for screening T-DNA knockout mutants in the P450 family gene. Because
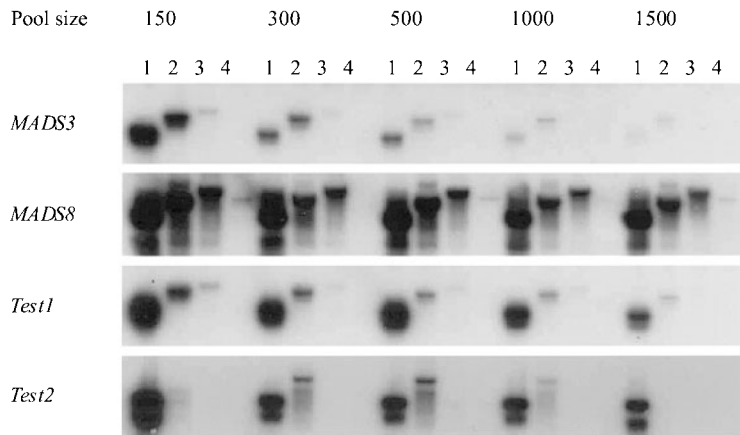
Pool size   150   300   500   1000   1500

1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4

MADS3

MADS8

Test1

Test2

Fig. 2 Screening efficacy according to various pool sizes. DNA pools of 150, 300, 500, 1,000, and 1,500 lines were PCR-amplified, with the T-DNA specific primer and gene-specific primers located about 1, 2, 3, and 4 kb from the T-DNA insertion sites. For all PCR reactions, 50-ng-pool templates were used.

the rice genome is about four times larger than for *Arabidopsis*, a pool of 500 lines was deemed sufficient for PCR-based screening in rice. Sato et al. (1999) have also used pools of ~550 lines to isolate a *Tos17* insertion in *OSH15*. Therefore, our results (Fig. 2) were consistent with these previous observations that a pool size of 500 to 1,000 was adequate when screening for a knockout mutant within the 2-kb targeting range.

After surface sterilization, 15 seeds from each individual line were germinated on soil, and DNA was extracted from the leaves of 10- to 14-day-old seedlings. We then made DNA from the subpools of an average of 90 lines. About 15 g of fresh leaves were harvested for each subpool; DNA yield was about 0.1 mg per gram of tissue. As shown in Table 1, a total of 235 subpools were created: 104 from the pGA2707-tagged lines for gene trap using the GUS reporter (Jeong et al. 2002), 60 from the pGA2715-tagged lines for activation tagging (Jeong et al. 2002), and 71 from the pGA2717-tagged lines for gene trap using the GFP and GUS reporters (unpublished data). Five subpools were combined to make a pool.

To determine the efficacy of the DNA pools, we selected the MADS-box genes present in rice by searching for DNA fragments that contained the conserved MADS-box domain from the public rice sequence databases registered in NCBI, TIGR, and rice GD (http://www.ncbi.nlm.nih.gov/, http://www.tigr.org/tdb/tgi/ogi/, http://btn.genomics.org.cn/rice/). This search resulted in the identification of 34 non-redundant MIKC-type MADS-box genes, which contain the domain adjacent to the putative start codon ATG and K-box (Table 2). For

comparison, 39 non-redundant MIKC-type MADS-box genes have been identified in *Arabidopsis* by complete genome-wide analyses (Parenicova et al. 2003).

A phylogenic tree of the 34 MIKC-type proteins from rice and 39 from *Arabidopsis* is shown in Fig. 3. These genes can be divided into 11 groups, based on sequence and functional homologies. Some of the A-, B-, and C/D-group MADS-box genes are well-known regulators of floral organ identity (Chung et al. 1995, Jeon et al. 2000b, Kang et al. 1995, Kang et al. 1998, Kyozuka and Shimamoto 2002, Nagasawa et al. 2003). Rice possesses four A-group genes (*OsMADS14*, *OsMADS15*, *OsMADS18*, and *OsMADS20*), three B-group genes (*OsMADS2*, *OsMADS4*, and *OsMADS16*), and three C/D-group genes (*OsMADS3*, *OsMADS13*, and *OsMADS21*). In addition, the three B sister (Bs)-group genes (*OsMADS29*, *OsMADS30*, and *OsMADS31*) identified in rice are homologous to the *Arabidopsis ABS* gene that is involved in normal endothelium development as well as seed pigmentation in the endothelium body (Nesi et al. 2002). Finally, five rice MADS-box genes (*OsMADS1*, *OsMADS5*, *OsMADS7*, *OsMADS8*, and *OsMADS34*) belong to the SEP group while two (*OsMADS6* and *OsMADS17*) are part of the AGL6 group. All of these belong to groups of genes known to be expressed preferentially in the reproductive organs, thereby suggesting a major role in floral and seed development.

On the other hand, the MADS-box genes in the remaining groups are expressed in vegetative tissues (Shinozuka et al. 1999). Four rice genes (*OsMADS26*, *OsMADS33*, *OsMADS35*, and *OsMADS36*) belong to the AGL12 group, which also con-

Table 1 DNA pools generated from the T-DNA insertion lines

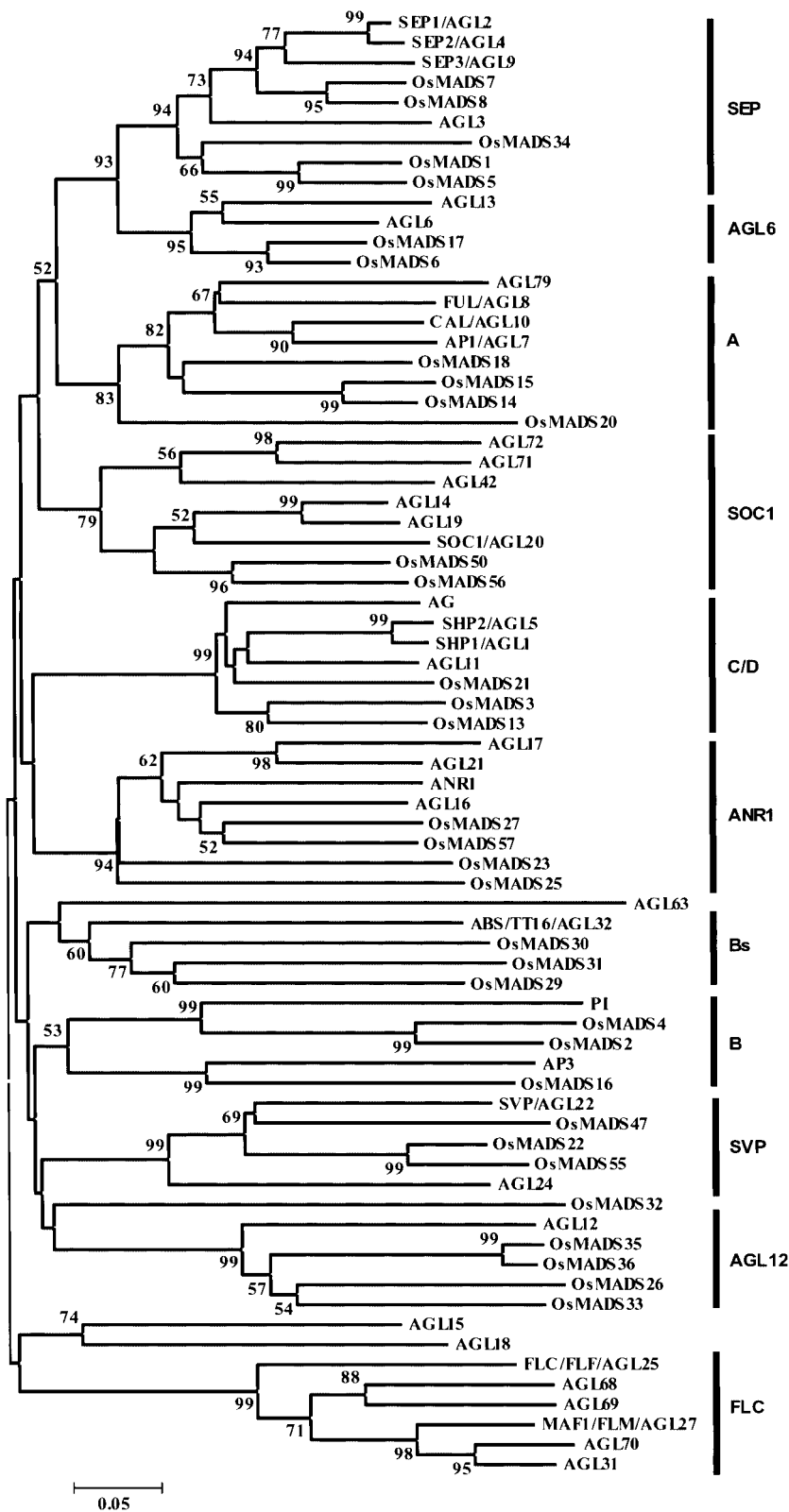|	Tagging vectors			
	pGA2707	pGA2715	pGA2717	Total
Number of pools	21	12	14	47
Number of subpools	104	60	71	235
Number of individuals	4,357	9,784	6,908	21,049

Fig. 3 Unrooted phylogenic tree of the MIKC type MADS-box genes. MADS-box protein sequences from rice and *Arabidopsis* were aligned using the program MAFFT (Katoh et al. 2002). The neighbor-joining (NJ) tree was constructed via the program MEGA2 (Kumar et al. 2001) with p-distance, complete deletion of gaps, and 500 bootstrap resamplings. Numbers beside each branch are percentiles of the resamplings. The scale bar indicates the number of amino acid substitutions per site.

Table 2 The MADS box genes in rice

Gene [a]	Genomic sequence accession number	cDNA accession number [c]	Protein size (a.a.)	Exon number	Chromosome	Knockout screening [d]	Reference
OsMADS1	AF204063	L34271	257	8	3		Chung et al. 1994
OsMADS2	AP003561	L37526	209	7	1		Chung et al. 1995
OsMADS3	AP003105	L37528	236	8	1		Kang et al. 1995
OsMADS4	AC109595	L37527	210	7	5		Chung et al. 1995
OsMADS5	AB026295	U78890	225	8	6		Kang and An 1997
OsMADS6	AP004178	U78782	250	8	2		Moon et al. 1999b
OsMADS7	AP005529	U78891	249	8	8		Kang et al., 1997b
OsMADS8	Contig1517 [b]	U78892	248	8			Kang et al., 1997b
OsMADS13	AL513004	AF151693	270	7	12		Lopez-Dee et al. 1999
OsMADS14	AF377947	AF058697	246	8	3		Moon et al. 1999b
OsMADS15	AP004342	AF058698	267	8	7		Moon et al. 1999b
OsMADS16	AP004329	AF077760	223	7	6		Moon et al. 1999a
OsMADS17	AL606688	AF109153	249	8	4		Moon et al. 1999b
OsMADS18	AP005175	AF091458	249	8	7		Moon et al. 1999b
<u>*OsMADS20*</u>	Scaffold1920 [b]	<u>AY250075</u>	233	7		0	This study
<u>*OsMADS21*</u>	AP003379	<u>AY177693</u>			1	0	This study
OsMADS22	AP004159	AB003322	229	8	2		Pelucchi et al. 2002
<u>*OsMADS23*</u>	AP003868	<u>AY345220</u>	159	5	8	0	This study
<u>*OsMADS25*</u>	AL731609	<u>AY177695</u>	227	7	4	0	This study
OsMADS26	AP004566	AB003326	222	7	8		Pelucchi et al. 2002
<u>*OsMADS27*</u>	AP004766	<u>AY177696</u>	240	6	2	0	This study
<u>*OsMADS29*</u>	AP004113	<u>AY177697</u>			2	0	This study
<u>*OsMADS30*</u>	AP003633	<u>AY174093</u>	221	7	6	2	This study
<u>*OsMADS31*</u>	AL731610				4		This study
OsMADS32	AP003343	AY177699	196	7	1	1	This study
<u>*OsMADS33*</u>	AL513004	<u>AY177700</u>	202	7	12	0	This study
OsMADS34	AF377947	AB003324	239	8	3		Pelucchi et al. 2002
<u>*OsMADS35*</u>	Contig40982 [b]						This study
<u>*OsMADS36*</u>	Contig16005 [b]						This study
OsMADS47	AC125471	AY345221	244	7	3		This study
OsMADS50	AC098695	AB003328	230	7	3		Shinozuka et al. 1999
<u>*OsMADS55*</u>	AP004322	<u>AY345223</u>	245	8	6	0	This study
OsMADS56	AC092697	AY345224	230	7	10	1	This study
<u>*OsMADS57*</u>	AP005751	<u>AY177702</u>	241	5	2	0	This study

[a] MADS-box genes identified in this study are underlined.

[b] China contig or scaffold numbers.

[c] GenBank accession no. Newly identified cDNA are underlined. Full-ORF cDNA of *OsMADS20*, *OsMADS23*, *OsMADS25*, *OsMADS27*, *OsMADS47*, *OsMADS55*, and *OsMADS57* were isolated from mixed libraries made from roots, sheaths, shoot apical meristems, panicles, and seed coats. In addition, full-ORF cDNA of *OsMADS30*, *OsMADS32*, and *OsMADS33* were isolated from the mixed cDNA prepared from calli, 2-week-old seedlings, mature leaves, young panicles (<5 cm), mature panicles, and developing seeds.

[d] Number of knockout mutants isolated from screening the 12 selected MADS-box genes.

tains one *Arabidopsis* MADS-box gene. These genes may be diversified for elaborate control of rice development. Another two rice genes (*OsMADS50* and *OsMADS56*) belong to the SOC1 group. Interestingly, this group is more diversified in *Arabidopsis*, with six MADS-box genes being represented there. Three rice genes (*OsMADS22*, *OsMADS47*, and *OsMADS55*) belong to the SVP group, while four ANR1-group genes (*OsMADS23*, *OsMADS25*, *OsMADS27*, and *OsMADS57*)

are also present in the rice genome sequence databases. Finally, four orphan MADS-box genes (*OsMADS32*, *AGL15*, *AGL18*, and *AGL63*) have been found, with the first being unique to rice and the others, to *Arabidopsis*. The FLC-group MADS-box genes are not identified in rice (Fig. 3).

Among the 34 rice MIKC-type genes, full-length cDNA clones for 19 are present in GenBank (Table 2). In addition, partial clones of *OsMADS32* and *OsMADS47* have been regis-

tered in the EST databases. Therefore, we newly identified 13 genes in this study. RT-PCR was then performed to examine whether they are functional. For our first analysis, we conducted experiments using mixed cDNA prepared from calli, 2-week-old seedlings, mature leaves, young panicles (<5 cm), mature panicles, and developing seeds. This resulted in the identification of 10 expressed MADS-box genes—*OsMADS20*, *OsMADS21*, *OsMADS23*, *OsMADS25*, *OsMADS27*, *OsMADS29*, *OsMADS30*, *OsMADS33*, *OsMADS55*, and *OsMADS57* (Table 2). We then investigated their expression patterns in four organs: seedling roots and shoots, young panicles, and developing seeds (Fig. 4). Five genes (*OsMADS27*, *OsMADS30*, *OsMADS33*, *OsMADS55*, and *OsMADS57*) were expressed ubiquitously, whereas three others were detected in only one organ type. Transcripts of the *OsMADS21* and *OsMADS29* genes were observed in developing seeds and the *OsMADS25* transcript was measured in the roots. *OsMADS20* transcript was detected in both shoots and seeds, while the *OsMADS23* transcript was found in roots and seeds. Transcripts of three MADS-box genes (*OsMADS31*, *OsMADS35*, and *OsMADS36*) were not detected, probably because they either are expressed in tissues not examined here or are inducible only under certain conditions. Alternatively, some of them may not be functional.
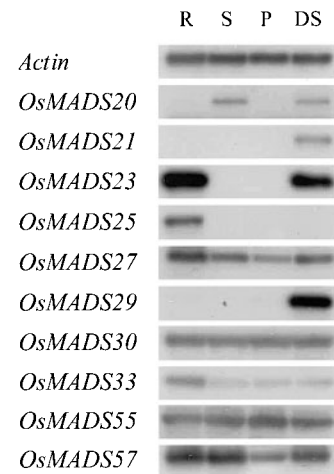

Fig. 4 Expression patterns of uncharacterized MADS-box genes. Total RNAs were isolated from seedling roots at 5 days post-germination (R), seedling shoots at 5 days after germination (S), panicles between 5 and 10 cm (P), and developing seeds (DS), using the RNA isolation kit (Tri Reagent; MRC Inc., Cincinnati, OH, U.S.A.). For RT-PCR, the first-strand cDNAs were synthesized from 2 μg total RNAs after DNase treatment, using M-MLV reverse transcriptase (Promega, Madison, WI, U.S.A.). RT-PCR analyses were performed with cDNA templates.
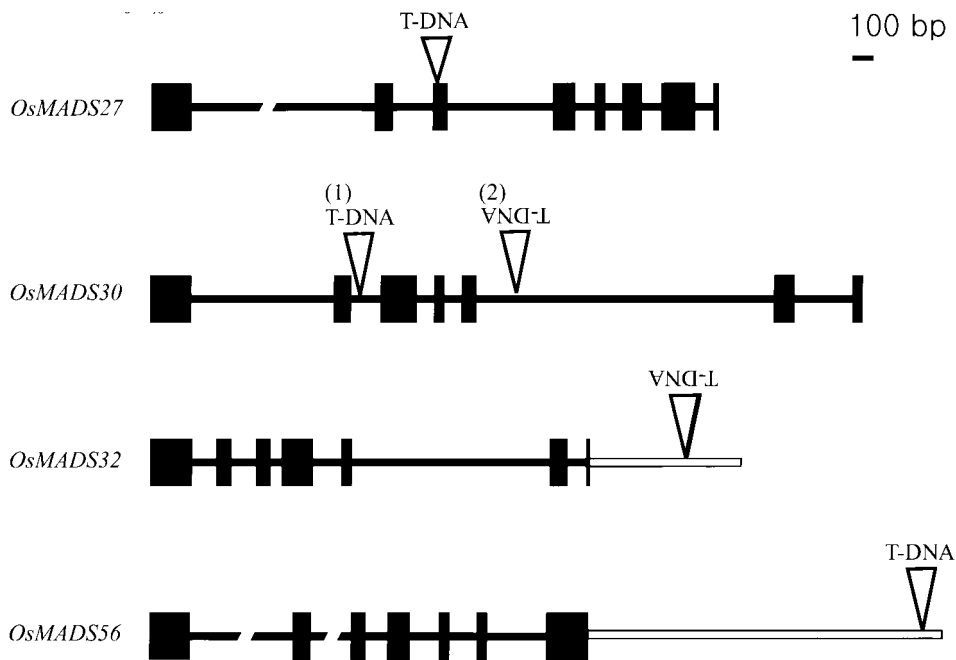

Fig. 5 T-DNA insertions in rice MADS-box genes were screened by PCR, using the pool DNAs as templates plus gene-specific primers and T-DNA primers: the right border primer, ATCCAGACTGAATGCCCACAGG, and the left border primers, CGATTTTTGAAATGCGAGAGCG (for pGA2707), ATCTTGAACGATAGCCTTTCCTTTATCG (for pGA2715), and GTCGGCCATGATATAGACGTTGTG (for pGA2717). PCR was performed under standard conditions using 50-ng pools as templates. Products were separated on 1% agarose gel, transferred to a nylon-membrane, and hybridized with gene-specific probes. When a hit was identified from a pool, subsequent PCR analysis of the subpools resulted in isolation of insertion positions in the MADS-box genes. PCR bands were sequenced to locate insertion sites, which are represented by open triangles above the schematic diagram of the MADS-box genes. Filled boxes indicate exons; filled bars, introns; open bars, regions outside the last exons; interruptions, long introns.

At the global level, expression patterns of the newly identified rice genes were similar to those of homologous *Arabidopsis* MADS-box genes. Rice *ANR1* homologs (*OsMADS23*, *OsMADS25*, and *OsMADS27*) were expressed in roots, similar to the patterns of *Arabidopsis ANR1*, *AGL17*, and *AGL21* (Burgeff et al. 2002, Rounsley et al. 1995). Except for *OsMADS25*, these genes were also detected in developing seeds, c.f. *ANR1* and *AGL21* (Burgeff et al. 2002). Interestingly, Alvarez-Buylla et al. (2000) have reported that another ANR1-group gene, *AGL16*, shows a higher level of expression in stems and rosette leaves than in roots and siliques, a pattern similar to that found here with *OsMADS57*.

In the case of the Bs-group genes, the *OsMADS29* transcript was detected only in developing seeds while that of *OsMADS30* was present in all organs. The profile of the former was similar to the *Arabidopsis* homolog *ABS*, which is expressed in both flowers and seeds, but not in vegetative tissues (Nesi et al. 2002). Just as with *Arabidopsis AGL12* (Rounsley et al. 1995), the *OsMADS33* gene was expressed preferentially in the roots. Moreover, the SVP homolog, *OsMADS55*, was ubiquitous, similar to the activity of *SVP* or *AGL24* in *Arabidopsis* (Hartmann et al. 2000, Michaels et al. 2003, Yu et al. 2002a). However, no transcripts of either *OsMADS20* (A-group) or *OsMADS21* (C/D-group) were detected in the developing panicles (i.e. 5–10 cm long). These results contradict the reported patterns of other genes in those two groups, where expression is found in the developing flowers (Flanagan et al. 1996, Kang et al. 1995, Kyozuka et al. 2000, Lopez-Dee et al. 1999, Rounsley et al. 1995, Savidge et al. 1995, Yanofsky et al. 1990).

We attempted to identify knockout mutants in two uncharacterized MADS-box genes (*OsMADS32* and *OsMADS56*) as well as the 10 newly identified genes whose transcripts were detectable. In all, five T-DNA insertions were identified in four genes (Fig. 5), and two independent insertions were found in *OsMADS30*. The T-DNA insertions in *OsMADS32* and *OsMADS56* occurred outside of those genes (538 bp and 1,591 bp downstream from the stop codon, respectively). Hence, expression may not have been affected by the insertions. In contrast, the T-DNA insertions in *OsMADS27* and *OsMADS30* likely influenced expression, because the T-DNA was large and contained at least one transcription terminator in either orientation.

In conclusion, we have now generated DNA pools from 21,049 T-DNA tagged lines, and have demonstrated that these pools can be used for identifying insertional mutants in rice genes. If T-DNA inserts randomly into rice chromosomes, this means that approximately 20% of the genes can be tagged (Jeon and An 2001). We also showed that the chance of finding a T-DNA insertion in any given gene was higher than the estimated value, probably because T-DNA prefers genic regions. We are now increasing the size of our DNA pools, thereby enhancing the probability of finding knockout mutants for par-

ticular genes. Therefore, our research provides a systematic framework for bridging the genomic resource to functional analysis of rice genes. The DNA pool screening service and the mutant seeds are available to public upon request to genean@postech.ac.kr.

Acknowledgments

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